

AN OFFPRINT FROM

# Venomous Snakes

## Ecology, Evolution and Snakebite

---

*Edited by* R. S. THORPE

W. WÜSTER

*and*

ANITA MALHOTRA

*School of Biological Sciences  
University of Wales  
Bangor*



*Published for* THE ZOOLOGICAL SOCIETY OF LONDON

*by* CLARENDON PRESS · OXFORD

1997

---

## 12 The role of ecology in determining venom variation in the Malayan pitviper, *Calloselasma rhodostoma*

---

JENNIFER C. DALTRY, WOLFGANG WÜSTER and  
ROGER S. THORPE

### Synopsis

Intraspecific variation in snake venom composition is a widespread phenomenon with important implications for snakebite therapy, yet few attempts have previously been made to identify its cause. The venom from almost 100 Malayan pitvipers (*Calloselasma rhodostoma*), captured in 36 localities in Vietnam, Thailand, Malaysia and Java, was analysed by using isoelectric focusing (IEF). A clear pattern of geographic variation emerged in the IEF profiles of adult *Calloselasma*, which was compared to patterns predicted from hypothetical causes by using a partial Mantel test. This test simultaneously evaluated the importance of geographic proximity ('venom variation is influenced by gene flow'), phylogeny ('venom reflects phylogenetic relationships among populations') and an ecogenetic hypothesis ('venom varies in accordance with geographic variation in prey type'). The intra-specific phylogeny was reconstructed by PCR-enhanced RFLP analysis of the cytochrome *b* gene (mtDNA), while dietary variation was assessed from the faeces and stomach contents of hundreds of wild-caught and museum specimens. Overall venom variation was significantly associated with the relative contribution of amphibians, reptiles and endotherms (mammals, birds) to local diet ( $P < 0.0001$ ). When 13 variable IEF bands were tested in turn, most were significantly associated with diet and only one reflected phylogeny, thereby calling into question the frequent use of venom as a 'neutral' marker of phylogeny in snake systematics. The pattern of geographic variation in IEF profiles was significantly congruent with the variation in the venom's enzymatic effects, indicating adaptive differences in the snakes' ability to subdue and digest different prey. This species also exhibits significant ontogenetic and sexual variation in venom composition and diet. Studies of the venom of captive Malayan pitvipers indicate that the dietary association is genetically based rather than induced in the short term; venom composition and prey selection may have co-evolved to optimize foraging efficiency.

## Introduction

Intraspecific variation in venom composition has been detected in numerous snakes (reviewed by Chippaux, Williams & White 1991). As well as being of academic interest to studies of the evolution and ecology of venomous snakes, this phenomenon is of major applied importance to snakebite therapy. For example, diagnosis of the species responsible for a bite can be confounded by variation in symptomatology, and the antivenom prepared against one venom type may be substantially less effective against another. Snake venom also represents a valuable natural source of biochemicals for research and medicine (Mebs 1978; Russell 1983), and it is often useful to determine whether the components of interest are more abundant in the venom of certain individuals than others. Yet despite its far-reaching consequences, the underlying causes of intraspecific variation in snake venom have never been clarified. Literature concerning the biochemistry/pharmacology of venoms, and the systematics/ecology of snakes have tended to travel separate paths, but all of these disciplines need to be integrated to ascertain how variation arises.

The Malayan pitviper, *Calloselasma rhodostoma*, was selected as the species through which to investigate this question. This terrestrial viperid is the leading cause of venomous snakebite morbidity throughout much of South-East Asia (Warrell 1986). Although the fatality rate of untreated bites is relatively low, many victims suffer severe necrosis, often leading to permanent deformity and long-term economic hardship (Warrell 1986). Ironically, the venom of this snake has also served a beneficial role in healthcare as the source of ancrod (Arvin<sup>TM</sup>), used clinically as a defibrinating agent. There have been numerous studies of the biochemistry and pharmacology of *C. rhodostoma* venom (see Hardy 1990 for bibliography), but the potential influence of intraspecific variation has been largely ignored. However, there are indications of regional differences in the bite symptomatology of *C. rhodostoma* (Warrell 1986) and in the venom's immunological properties (Tu & Ganthavorn 1978), which suggests that venom composition varies geographically.

The causes of geographic variation can be elucidated with the aid of numerical hypothesis testing. A distance (similarity) matrix based on the observed pattern of variation in phenotype can be compared with matrices representing hypothesized causes by using Mantel tests to evaluate the probability of their association (see Manly 1985). A partial Mantel test is preferred because it evaluates rival hypotheses simultaneously, regressing out any effects of intercorrelation between them. This rigorous test has previously proven invaluable for determining the causes of geographic variation in reptiles (e.g. Thorpe 1991).

The aim of this paper is to provide a more precise evaluation of the pattern of variation in the venom of *C. rhodostoma* and to identify its most probable evolutionary cause through the use of partial Mantel tests. Although the primary focus will be geographic variation, within-population variation in venom composition will also be discussed.

## Materials and methods

### Venom collection and electrophoresis

Venom samples were obtained from 96 *C. rhodostoma* captured in 36 localities in Vietnam, Thailand, Malaysia and Java (Fig. 1). Venom was extracted within 12 h of capture and desiccated to prevent deterioration in storage (Tan & Tan 1987).

The samples were rehydrated with reverse osmosis water to a concentration of 10 mg/ml soluble protein, and compared by using IEF across polyacrylamide gels of pH range 3.5–9.5 (Ampholine PAGplates<sup>TM</sup> purchased precast from Pharmacia LKB). Fifteen microlitres of each venom solution were focused for 80 min at up to 1500 V, and stained with Coomassie Blue.

To determine whether venom composition changes with body size, a comparison was made of the IEF profiles of specimens from the best-represented region, West Java (groups 29–36;  $n = 20$ ), of snout–vent length (SVL) 274 to 626 mm. A 20 × 20 distance matrix derived from the occurrence of all varying bands was tested against SVL by a Mantel test with 10 000 randomizations.

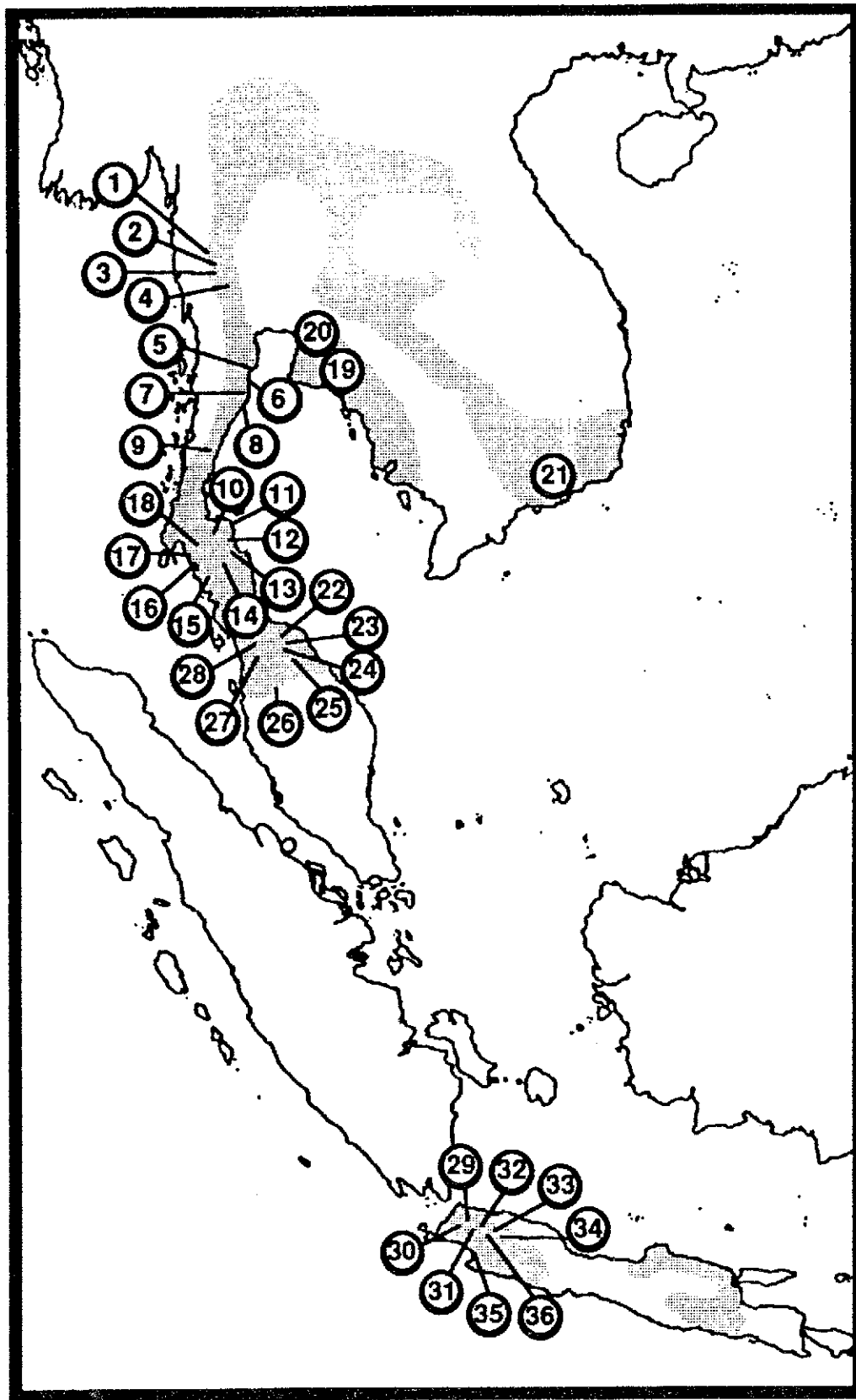
### Causal hypotheses

Three hypotheses to explain geographic variation in venom were tested:

#### 1. Variation in venom is associated with population phylogeny

Interspecific variation in venom is widely assumed to be a function of phylogeny, and similarities in venom properties between species (or higher order taxa) are often interpreted as evidence of common ancestry (e.g. Githens & George 1931; Foote & MacMahon 1977). If geographic variation in the venom of *C. rhodostoma* evolved in accordance with its population phylogeny, populations with a more recent common ancestor would be expected to produce more similar venom than phylogenetically remote populations.

The historical relationships among populations are most reliably inferred (as free as feasible from ecogenetic selection) by comparing their mitochondrial DNA (mtDNA) (Avice 1994). A few millimetres of caudal tissue were collected from each snake after venom extraction. Biopsies were also taken from other specimens obtained in the field, to give a total of 131 samples. From the tissue, 767 bp fragments of mtDNA, chiefly the cytochrome *b* gene (709 bp), were amplified by means of the polymerase chain reaction using the primers L14841' (Kocher *et al.* 1989) and MVZ16 (Moritz, Schneider & Wake 1992). Seven restriction endonucleases (*DdeI*, *EcoRI*, *HinfI*, *NciI*, *NlaIII*, *ScrFI*, *TaqI*) identified 19 different haplotypes among the 131 mtDNA samples. Genetic distances between the populations were computed with program DA, REAP (McElroy *et al.* 1992) and used to construct a Fitch–Margoliash phylogenetic tree (program KITSCH, PHYLIP 3.3: Felsenstein 1991) (Fig. 2). Three main lineages were resolved: (1) north-western Thailand; (2) Vietnam with the rest of Thailand; and (3) Malaysia with Java.



**Fig. 1.** Sites from which venom samples were collected: the Thai provinces of Kanchanaburi (sites 1–4); Prachuap Khiri Khan (5–8); Chumphon (9); Nakhon Si Thammarat (10–14); Krabi (15–18); Rayong (19, 20); Vung Tau province, Vietnam (21); Kedah State, Malaysia (22–28); West Java, Indonesia (29–36). Shading represents the known distribution range of *C. rhodostoma*.

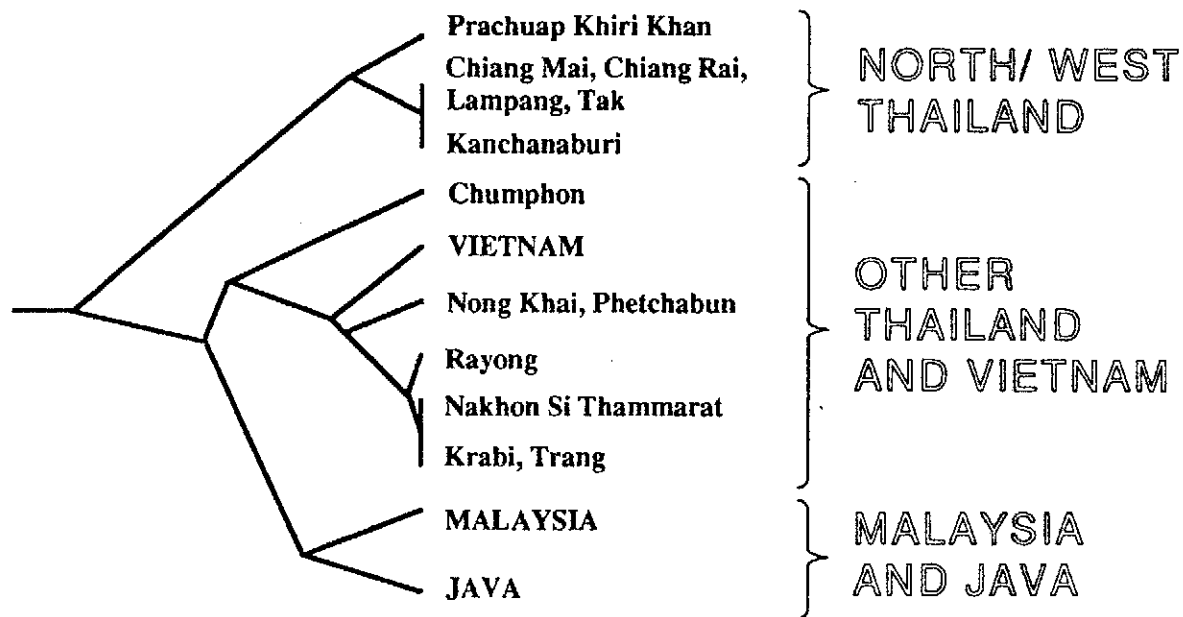


Fig. 2. Population phylogeny of *C. rhodostoma* reconstructed from RFLP analysis of the cytochrome *b* gene. Branch lengths are drawn to scale.

## 2. Variation in venom is associated with the geographic variation in diet

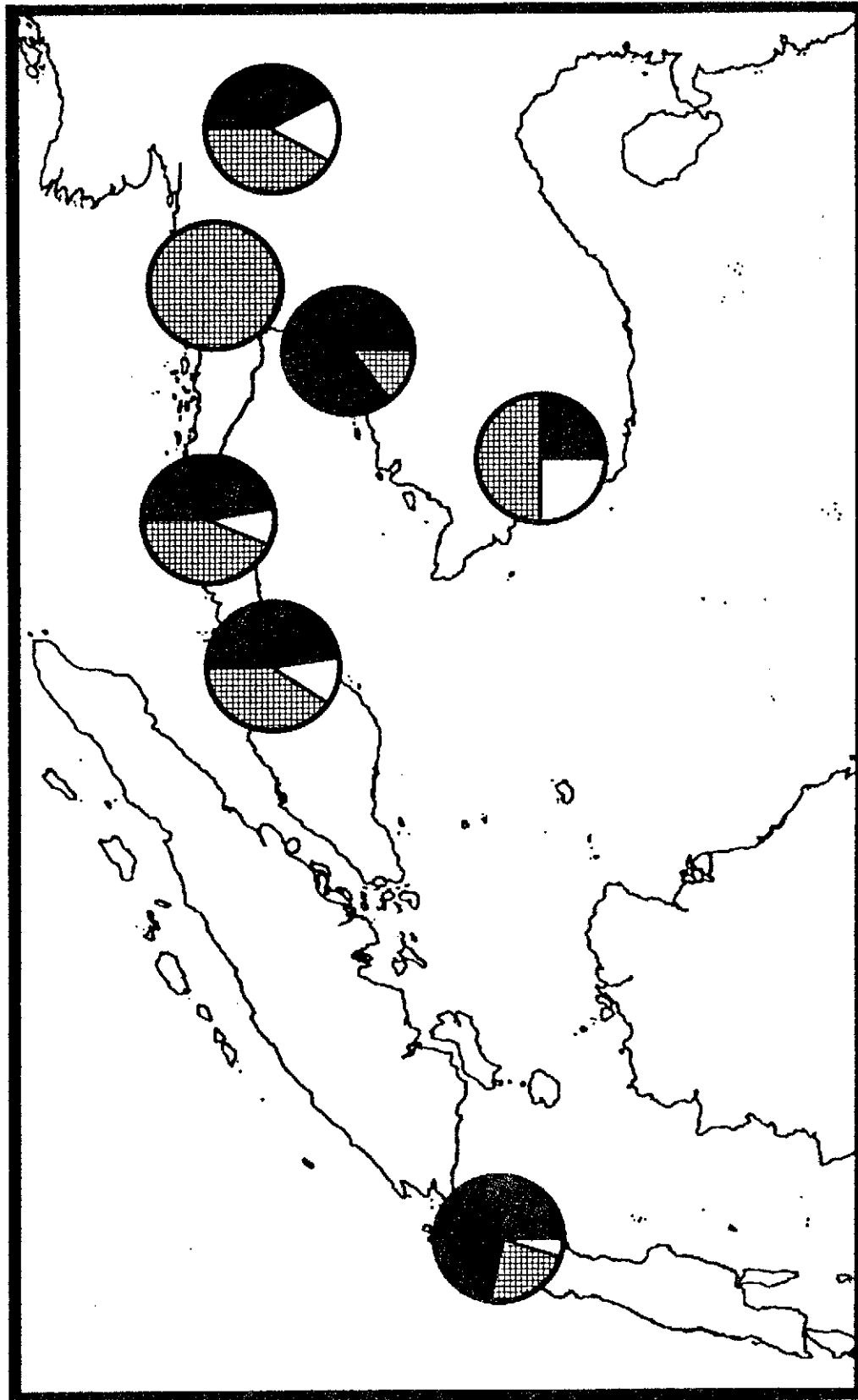
Since venom plays an important role in feeding (Thomas & Pough 1979; Russell 1983; Mackessy 1988), venom variation might be linked to dietary variation. Diet was recorded from the faeces of the specimens that provided venom, supplemented by the gut contents of museum and roadkilled specimens from the same localities. Two hundred and sixteen prey items were identified to taxonomic class, including insects, chilopods, fish, amphibians, reptiles, rodents and birds. There was significant geographic variation in the contribution of different classes to the diet of adults (Fig. 3).

## 3. Variation in venom is a function of the geographic distance between groups

Geographic proximity might influence venom evolution in several ways. Firstly, the opportunity for exchange of venom-coding genes tends to be higher between neighbouring demes and may enable spatially-close populations to produce more similar venom than remote conspecifics; secondly, neighbouring populations are more likely to share a similar habitat, which might promote convergent evolution of venom.

### Numerical hypothesis testing

To evaluate the causes of geographic variation, the venom profiles of adult *C. rhodostoma* of > 400 mm SVL were segregated into 36 groups according to capture locality (Fig. 1). (Smaller specimens were excluded to remove the ontogenetic influence on venom composition, and the gender influence was removed by excluding bands of pI 5.90 and 6.90: see Results.) Each regional group was given a series of digits denoting the mean occurrence of each band (e.g. 0 = band absent from all group members; 0.5 = present in 50% group members).



**Fig. 3.** Geographic variation in the diet of adult *C. rhodostoma*. The pie charts represent the contribution of amphibians (white), reptiles (cross-hatched) and endotherms (black) as a percentage number of items identified from specimens larger than 400 mm SVL.

Observed variation in venom among the groups was expressed as a  $36 \times 36$  distance matrix. A partial Mantel test was used to partially correlate the observed matrix against three matrices derived from the three hypotheses above. (1) The

phylogenetic relationships among the 36 groups were assessed by RFLP analysis of mtDNA, and patristic distances were computed between each pair of groups by using a Fitch–Margoliash phylogenetic tree (program KITSCH, PHYLIP 3.3: Felsenstein 1991) constructed from genetic distances calculated with program DA (REAP: McElroy *et al.* 1992). By regressing out the patristic distances between populations, the partial Mantel test makes it possible to test for ecogenetic adaptation free of phylogenetic effects (Thorpe *et al.* 1995). (2) Diet was recorded as the mean proportions of amphibians, reptiles and endotherms (birds, mammals) constituting the diet of snakes exceeding 400 mm SVL in each region (see Fig. 3). (3) Geographic proximity was computed from the latitude and longitude of the 36 localities.

In the first partial Mantel test, the dependent matrix was based upon *all* variable venom bands and thus represents the overall similarities between the groups. However, since there is no reason to suppose that all venom components evolve in the same way, subsequent tests compared the geographic variation in each band in turn with the three hypotheses of phylogeny, diet and geographic proximity.

The association between venom and diet was also tested by the comparative method of independent contrasts (Felsenstein 1985). The continuous variables representing venom and diet variation were taken as the largest principal coordinate of the venom and diet matrices respectively, and entered into Garland's Comparative Method Analysis Package (CMAP) under the gradual change model. This approach is commonly used to elucidate evolutionary cause, but is inferior to the partial Mantel test for studying variation within species because it cannot take into account gene flow between populations (estimated from geographic proximity) or other multi-dimensional hypotheses. Furthermore, the comparative method does not, on its own, consider multiple competing hypotheses free of distribution.

## Results

### The patterns of variation in venom composition

About 25 intense bands and numerous trace bands were resolved per isoelectrically focused venom sample. For comparison, only intense bands (the most abundant components) will be considered.

Ontogenetic variation was evident in all regions where both adults and juveniles had been sampled; for example, 11 bands varied among the venoms from West Java ( $n = 20$ ), and occurrence was significantly associated with body size ( $P = 0.0190$ ).

Owing to this influence of body size, only venoms from adult specimens exceeding 400 mm SVL ( $n = 67$ ) were compared to determine the pattern of geographic variation. Across these venoms, bands representing 37 different isoelectric points were identified, of which 19 were not present in all samples. Two of these were gender-specific. Adult females from west Java, Malaysia and west and peninsular Thailand ( $n = 19$ ) produced a dark band of pI 6.90 that was absent from all males in these regions ( $n = 19$ ). Two males from south-east Thailand also lacked this band, but no data is available on the venom of local females. In Vietnam, both genders lacked band pI 6.90, but every female ( $n = 9$ ) produced a dark band of pI 5.90 which was absent from Vietnam males ( $n = 6$ ).

Each specimen was given a score of 17 digits (denoting the occurrence of the remaining 17 variable bands) which was entered into a principal component analysis (Fig. 4). Fifty-three variants were identified (specimens with identical venoms invariably came from the same locality). The first two component scores (PC1, PC2) account for 42.64% of the total variation.

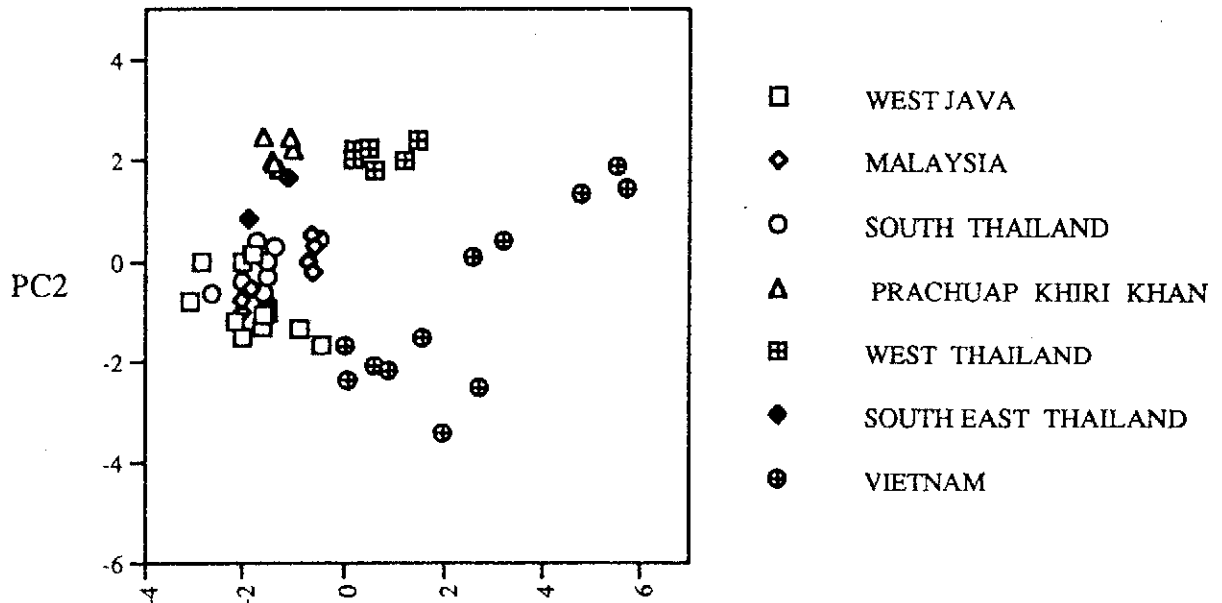


Fig. 4. Principal components analysis of isoelectrically focused electrophoretograms of venom from adult *C. rhodostoma*.

#### Partial Mantel tests

When venoms from 67 adult *C. rhodostoma* were grouped by capture locality (Fig. 1), 13 bands varied in occurrence among the 36 groups.

Geographic variation in overall venom composition was not significantly associated with geographic distance or patristic distance, but *was* significantly correlated to the proportions of amphibians, reptiles and mammals constituting local diet (Table 1). Gene flow and phylogenesis must therefore be dismissed as primary influences, but we cannot reject the hypothesis that venom variation is in some way linked to diet.

When the variable bands were tested in turn, almost half were significantly associated with diet, two with geographic proximity and only one with population phylogeny. Note that even when other tree-building algorithms (Fitch–Margoliash without a molecular clock assumption, neighbour-joining, and parsimony or maximum likelihood using modal haplotypes) were used to reconstruct the population phylogeny, the results were still as strongly, and in some cases more strongly, in favour of diet.

#### Independent contrasts method

The findings of the Mantel tests were supported by the independent contrast method. The correlation between diet and venom, free of phylogenetic effects, was highly significant ( $r = 0.67$ ;  $P < 0.01$ ).

**Table 1.** Probabilities of significance associated with partial Mantel tests comparing geographic variation in venom with causal hypotheses

	Patristic	Diet distance	Geographic proximity
Overall venom composition	0.0225	<0.0001*	0.5358
Band pI 9.40	0.7012	0.0046	0.0576
Band pI 9.15	0.0018	0.0506	0.0009*
Band pI 8.55	0.1680	0.1023	0.0041
Band pI 8.45	0.2217	0.3542	0.6542
Band pI 8.25	0.6280	0.0011*	0.0083
Band pI 7.85	0.0862	0.4000	0.6182
Band pI 7.45	0.0015*	0.7296	0.8052
Band pI 6.55	0.6700	0.0012*	0.0321
Band pI 6.10	0.6429	0.7233	0.1113
Band pI 6.00	0.0160	0.0624	0.0009*
Band pI 5.80	0.4829	0.0012*	0.0542
Band pI 5.70	0.4727	0.0002*	0.0247
Band pI 5.45	0.4944	0.0006*	0.0044

\*  $P < 0.05$  after applying sequential Bonferroni correction (Rice 1989) to all tests in the table.

## Discussion

### The biological function of the venom of *C. rhodostoma*

Before attempting to interpret the partial Mantel test results, it is useful to consider the biochemistry and biological role of the venom of *C. rhodostoma*.

The primary roles of the venom of most snakes are to subdue prey and initiate prey digestion (Russell 1983). Snakes in general, and the Viperidae in particular, consume relatively large prey (Pough & Groves 1983); *C. rhodostoma*, for instance, commonly tackles prey equalling one third of its body weight, including rats and scolopendrid centipedes (Daltry 1995). Such formidable prey would undoubtedly be more difficult, even hazardous, to subdue without the aid of venom.

It is also crucial that such large meals are digested quickly enough to prevent the prey's gut bacteria from causing excessive putrefaction. Digestive enzymes produced in the stomach can only permeate the prey via its largely intact epidermis (which may be shielded by scales, fur or feathers), whereas enzymes in the venom are injected below the dermis, and are dispersed by diffusion and the prey's circulatory system. In accordance with their proclivity for large prey, the Viperidae have long fangs; a *C. rhodostoma* 1 m long, for example, has a penetration depth of 16 mm. Long fangs do not necessarily improve the efficiency of venom delivery (Hayes, Kaiser & Duvall 1992), but do ensure that digestive enzymes are released deep within the prey.

The venoms of various viperid snakes have been shown to have a powerful digestive effect (Thomas & Pough 1979). A major constituent of these venoms is proteolytic enzymes, which cause the breakdown of structural proteins in the

muscles, organs and subcutaneous tissue. The venom of *C. rhodostoma* contains several different basic proteases and exhibits exceptionally high L-amino acid oxidase activity (Tan 1991), which is implicated in protease activation (see Mackessy 1988). Other known digestive components are reviewed by Tan (1991).

The toxicity of *C. rhodostoma* venom, on the other hand, is relatively low (see Mebs 1978). Large prey are often released after striking and it is essential that they should be rapidly immobilized by the venom before fleeing too far or injuring the snake, but it is not necessary or even advantageous to cause rapid death since the venom digestive enzymes may be usefully dispersed by the prey's circulatory system. Mice injected with small doses of *C. rhodostoma* venom rapidly lose locomotory ability, but death is protracted and accompanied by nervous spasms (Plagnol & Martin 1957), which may benefit the snake by ensuring thorough dissemination of the venom (Mackessy 1988).

#### **Causes of geographic variation in venom**

IEF revealed a strong pattern of geographic variation in *C. rhodostoma* venom (Fig. 4). Interestingly, this pattern is significantly congruent with variation in the venom's enzymatic activities (Daltry 1995); that is, venoms with similar IEF profiles have similar biological effects.

The overall pattern of variation in venom composition was significantly correlated to the diet of adults (Table 1). This might be interpreted as evidence of environmental induction. However, venoms collected from captive-bred *C. rhodostoma* closely matched those of wild specimens from the native origin of the captive stock, even though the all-mouse diet of the captives is incongruent with the more varied natural prey. Conversely, intraspecific variation is often evident even in the venom of captives maintained on the same diet (Bonilla, Faith & Minton 1973; Gregory-Dwyer *et al.* 1986). These observations, in addition to findings that hybrid snakes produce a venom exhibiting characteristics of both parents (e.g. Aird *et al.* 1989), suggest that venom variation is predominantly dictated by fixed, heritable genes.

A more plausible explanation for the association between diet and venom is evolution through natural selection. Pitvipers may specialize on certain prey, according to which are locally abundant or obtained with least 'cost', and selection will favour the individuals producing the most effective venom for subduing and ingesting these prey. The adaptive evolution of the venom might in turn promote increased dietary specialization, with the snakes focusing upon the animals that are most efficiently handled by means of their venom. (It is not yet clear whether the variation in diet is primarily a passive response to variation in local availability of prey types, or reflects inherent differences in prey 'choice'. Congenital variation in prey selection has been demonstrated in other snakes: see Arnold 1981.)

Of course, the hypothesis that venom is adapted to local diet hinges upon variation among prey species in susceptibility to venom. Indeed, venom lethality varies between taxa by orders of magnitude (Mebs 1978; Russell 1983). Prey

'digestibility' has less frequently been compared, but the digestive role of venom may be especially valuable where the animal is relatively large, has a low surface-area : volume ratio and/or has a resistant outer coating, since these attributes retard normal digestion in the stomach. Intuitively, the highly permeable dermis of a frog presents a lesser challenge to the stomach enzymes than a reptilian skin. Squamate reptiles have a high surface-area : volume ratio, however, and may therefore be digested more easily than a bulky rodent (Thomas & Pough 1979; Mackessy 1988). Taxonomic differences in membrane structure and physiology may also affect the efficacy of the venom. Consequently, specialization upon different prey could select for different quantities and types of toxins and digestive enzymes in the venom.

Snakes frequently exhibit geographic variation in diet (e.g. Arnold 1981; Shine 1991), and further research is necessary to determine whether this is commonly correlated with geographic variation in venom composition. We would suggest, however, that a venom-diet association will not be equally strong in all species. *C. rhodostoma* is common and widespread, and the fixation of venom-coding genes by different populations under natural selection is therefore unlikely to have been significantly perturbed by the vagaries of genetic drift. In species typified by small, isolated populations, on the other hand, the influence of drift can become accentuated (Mayr 1975), thereby reducing the likelihood of similar venoms being produced by different demes even if their diet is identical. Indeed, Williams *et al.* (1988) compared the venom electrophoretograms of insular tiger snakes (*Notechis ater*) and found that within-population variation is correlated with population size, suggesting that drift has reduced genetic diversity on small islands. Unlike *C. rhodostoma*, the geographic variation in the venom was *not* significantly associated with available prey.

#### **Ontogenetic and sexual variation**

Distinct banding differences were observed between the IEF profiles of venoms from juvenile and adult *C. rhodostoma* from the same localities. Ontogenetic changes in venom electrophoretograms do not necessarily reflect changes in biological activity, but this is frequently the case (e.g. Bonilla *et al.* 1973; Aird 1985; Andrade & Abe 1993). Marked changes in the effects of the venom are commonplace in viperid species (see Chippaux *et al.* 1991), and there is mounting evidence that they are adaptively associated with changes in diet. Juvenile *Crotalus viridis*, for instance, prey chiefly upon lizards and their venom is 2.5 times more toxic to lizards than the venom of the rodentivorous adults (Mackessy 1988). Generally speaking, mammalian prey can be subdued by a less toxic venom than reptile prey, but, being bulky, are difficult to digest. This might explain why the venom of adult *C. viridis* displays proteolytic activity five times higher than that of juvenile venom (Mackessy 1988). Many other venomous snakes undergo ontogenetic shifts in diet from ectothermic to endothermic prey (Klauber 1972; Sazima 1992; Andrade & Abe 1993) and concurrent changes to a more proteolytic but less toxic venom are widely reported (Chippaux *et al.* 1991).

In most regions, small *Calloselasma rhodostoma* prey chiefly upon ectotherms whereas adults show a stronger proclivity for endotherms (Daltry 1995), so it is possible that the venom of different age-groups is specifically adapted to their contrasting foraging requirements. Of course, an alternative, non-selective explanation is that the ontogenetic changes merely reflect unavoidable developmental constraints. However, not all snakes undergo distinct changes in venom; the venoms of juvenile and adult *Oxyuranus microlepidotus* (Elapidae) exhibit similar electrophoretic patterns and biological activities (Tan, Ponnudurai & Mirtschin 1993) and all age groups eat small mammals (Shine 1991). Similar conservatism of both venom and diet is also exhibited by *Crotalus durissus terrificus* in south-east Brazil (Salomão, Santos & Puerto 1995).

Irrespective of body size, female *C. rhodostoma* consistently produced high levels of a venom protein(s) that was not produced by local males. Sexual variation may be associated with diet since females tend to grow larger, have proportionally larger heads, and display a stronger proclivity for larger prey taxa (Daltry 1995). Yet even small females produce this extra band. Either there are more subtle gender differences in diet which have not yet been detected (our dietary analysis compared prey classes and further data are required regarding actual species), or the sexual variation, though widely conserved, has no adaptive function. After all, by definition, the sexes have an unequal distribution of sex chromosomes and hormones which may promote divergent development of non-debilitating (but not necessarily advantageous) secondary sexual characteristics.

Differences were also frequently apparent between venoms from pitvipers collected from the same locality on a similar date, of the same gender and similar body size. This probably reflects innate individual variation in venom synthesis, since genetic variation is to be expected in the gene pool of most sexually reproducing species.

#### **Potential implications for snakebite therapy**

Identification of the species responsible is often crucial for effective snakebite therapy, but few victims can reliably identify the snake responsible. Diagnosis of *C. rhodostoma* bite is typically founded upon the symptoms presented, but this may be confused by intraspecific variability in clinical effects. Geographic differences in the symptomatology of *C. rhodostoma* bites between Malaysia and Thailand were reported by Warrell (1986) and, given the ontogenetic, sexual and individual variation in venom composition shown herein, within-locality clinical variation is also likely (see Russell 1983).

Unfortunately, even when the species has been correctly identified, the appropriate antivenoms for bites by different populations or age groups may radically differ. Gutiérrez *et al.* (1991), for example, found that antivenom prepared against adult Costa Rican *Crotalus durissus durissus* was a poor neutralizer of local juvenile venom, which differs from the adult venom in this population, whereas in Brazilian *C. d. terrificus* it does not. In the case of *Calloselasma rhodostoma*, physicians treating bites in Malaysia suspect that antivenom prepared from Thai

populations is less effective than antivenom prepared from local specimens (S. Ambu pers. comm.).

Producers of *C. rhodostoma* antivenom typically obtain venoms from areas where the species is most easily collected and, since juveniles are hard to find and produce little venom, large specimens are favoured. Yet Chippaux *et al.* (1991) stated that 'producers of antivenom must . . . ensure representation of all venom types required within each antivenom'. In the case of *C. rhodostoma* venom, this approach might entail obtaining venom from specimens of both genders and various age-groups from across the geographic range in which the antivenom is to be used, which would inevitably raise the cost of antivenom production. It is therefore crucial to ascertain the extent to which the intraspecific variation in venom composition revealed in the present study reflects differences of importance to antivenom efficacy. After all, some of the components in the venoms might not be of pharmacological importance, while others may have serious clinical effects yet fail to stimulate antibody responses (Chinonavanig *et al.* 1988).

#### **Potential implications for biochemical research and snake systematics**

Venom proteins are frequently used as tools to investigate biological processes and structure (Mebs 1978; Russell 1983). Research using ancrod, for instance, has shed light on the processes of blood coagulation in humans (Denson 1969). A better understanding of intraspecific variation in quantity and types of venom components could help to pinpoint the optimal sources of the desired compound.

Chippaux *et al.* (1991) advised investigators of the biochemistry and biological activity of venom to monitor the precise origin of the sample under analysis, so that the findings can be replicated and interpreted in a biologically meaningful way. *C. rhodostoma* venom has been subjected to intense research (Hardy 1990), but almost every study has been based upon commercial sources of uncertain geographic origin, body size or gender. Perhaps inconsistencies in the venom source partly explain why experimental findings often vary widely among authors. The intravenous LD<sub>50</sub> of *C. rhodostoma* venom, for instance, ranges from 2.8 to 6.2 mg/kg mice (Mebs 1978).

Intraspecific variation in venom also has serious consequences for taxonomists. Given the special importance of accurate systematics among dangerous snakes (Wüster & Thorpe 1991), the means by which phylogenetic relationships are reconstructed require serious consideration. Venoms are frequently compared to elucidate taxonomic relationships, with compositional similarities usually interpreted as evidence of common ancestry (e.g. Githens & George 1931; Chen, Wu & Zhao 1984), but findings may vary according to the specimen(s) chosen to represent each taxon; for example, the venom of juvenile *Crotalus d. durissus* from Costa Rica is more similar to adult Brazilian *C. d. terrificus* than adult *C. d. durissus* (Gutiérrez *et al.* 1991).

Even when specimens of equal size are compared, there is no guarantee that their venoms will accurately reflect their phylogenetic affinities. The venom of adult *C. d. durissus*, for example, shows a greater immunological affinity with *C. adamanteus*

and *C. viridis* than with the conspecific *C. d. terrificus* (Anderson, Gutiérrez & Ownby 1993). Only one IEF band in adult *C. rhodostoma* venom reflected phylogeny (Table 1). Clearly, venom may not be a reliable phylogenetic marker, particularly at low taxonomic levels.

#### Is intraspecific variation in venom predictable?

Much remains to be learned of the patterns of inter- and intraspecific variation in venom composition and pharmacology of medically important snakes, but collecting and analysing venom from a broad geographic area is laborious, expensive and hazardous. Is it possible to predict where and to what extent variation is present? Regrettably, the findings of this study suggest it is not: diet was a better predictor of venom differences between populations of *C. rhodostoma* than either geographic proximity or phylogeny, but the prey of most species are too poorly known to predict venom variation.

Can venom variation instead be inferred from the snakes' appearance? Many studies have found differences in composition that *seem* to accord with the distribution of subspecies characterized by morphological features (Chippaux *et al.* 1991), but the comparisons were often tautologically made between pooled samples from the ranges of the nominal subspecies. Closer scrutiny reveals that the correlation between venom and morphology is less clear-cut; for example, the venom of *Crotalus lepidus klauberi* is usually similar to *C. l. lepidus* venom, but *C. l. klauberi* from Chihuahua and parts of Arizona and New Mexico produce a potent toxin and can be a hundred times more lethal (Rael *et al.* 1992).

*Calloselasma rhodostoma* also exhibits significant geographic variation in coloration, scalation and body shape, but it is impossible to identify different venom variants simply from such variation in appearance (Daltry 1995). It would seem that painstaking biochemical analysis is the only sure means of determining intraspecific variation in venom.

#### Acknowledgements

We thank the Zoo Negara (Malaysia) for venom, N.-H. Tan and G. Ponnudurai for enzymological assays, J. Norman for advice on molecular methods, B. J. F. Manly and T. Garland for programs, and the museums that loaned specimens. T. K. Anh and T. X. Kiem (Choray Hospital, Vietnam), S. Ambu and B. L. Lim (Institute for Medical Research, Malaysia) and C. K. Shin (Zoo Negara) provided stimulating discussions. This research was primarily funded by a SERC studentship (JCD) and the Leverhulme Trust (RST, A. Malhotra), with support from NERC (WW) and the Royal Society (RST).

#### References

- Aird, S. D. (1985). A quantitative assessment of variation in venom constituents within and between three nominal rattlesnake subspecies. *Toxicon* 23: 1000–1004.

- Aird, S. D., Thirkhill, L. J., Seebart, C. S. & Kaiser, I. I. (1989). Venoms and morphology of western diamondback/Mojave rattlesnake hybrids. *J. Herpet.* 23: 131-141.
- Anderson, S. G., Gutiérrez, J. M. & Ownby, C. L. (1993). Comparison of the immunogenicity and antigenic composition of ten Central American snake venoms. *Toxicon* 31: 1051-1059.
- Andrade, D. V. & Abe, A. S. (1993). Toxicidade do veneno de *Bothrops moojeni* e sua relação com a variação ontogenética de dieta. (Abstract). In *III congresso Latino Americano de herpetologia: 77*. Instituto de Biologia, Universidade Estadual de Campinas, Campinas, São Paulo.
- Arnold, S. J. (1981). Behavioral variation in natural populations. II. The inheritance of a feeding response in crosses between geographic races of the garter snake, *Thamnophis elegans*. *Evolution* 35: 510-515.
- Avise, J. C. (1994). *Molecular markers, natural history and evolution*. Chapman and Hall, London.
- Bonilla, C. A., Faith, M. R. & Minton, S. A. (1973). L-amino acid oxidase, phosphodiesterase, total protein and other properties of juvenile timber rattlesnake (*Crotalus b. horridus*) venom at different stages of growth. *Toxicon* 11: 301-303.
- Chen, Y., Wu, X. & Zhao, E. (1984). Classification of *Agkistrodon* species in China. *Toxicon* 22: 53-61.
- Chinonavanig, L., Billings, P. B., Matangkasombut, P. & Ratanabanangkoon, K. (1988). Antigenic relationships and relative immunogenicities of venom proteins from six poisonous snakes of Thailand. *Toxicon* 26: 883-890.
- Chippaux, J.-P., Williams, V. & White, J. (1991). Snake venom variability: methods of study, results and interpretation. *Toxicon* 29: 1279-1303.
- Daltry, J. C. (1995). *The evolutionary biology of the Malayan pit viper: a study of the causes of intraspecific variation*. PhD thesis, University of Aberdeen.
- Denson, K. W. E. (1969). Coagulant and anticoagulant action of snake venoms. *Toxicon* 7: 5-11.
- Felsenstein, J. (1985). Phylogenies and the comparative method. *Am. Nat.* 125: 1-15.
- Felsenstein, J. (1991). *PHYLIP, version 3.3*. University Herbarium, University of California, Berkeley.
- Foote, R. & MacMahon, J. A. (1977). Electrophoretic studies of rattlesnake (*Crotalus* and *Sistrurus*) venom: taxonomic implications. *Comp. Biochem. Physiol. (B)* 57: 235-241.
- Githens, T. S. & George, I. D. (1931). Comparative studies on the venoms of certain rattlesnakes. *Bull. Antivenin Inst. Am.* 5: 31-34.
- Gregory-Dwyer, V. M., Egen, N. W., Bosisio, A. B., Righetti, P. G. & Russell, F. E. (1986). An isoelectric focusing study of seasonal variation in rattlesnake venom proteins. *Toxicon* 24: 995-1000.
- Gutiérrez, J. M., dos Santos, M. C., Furtado, M. F. & Rojas, G. (1991). Biochemical and pharmacological similarities between the venoms of newborn *Crotalus durissus durissus* and adult *Crotalus durissus terrificus* rattlesnakes. *Toxicon* 29: 1273-1277.
- Hardy, D. L. (1990). Venoms and envenomation: a selected bibliography of the recent literature on *Agkistrodon* and its allies. In *Snakes of the Agkistrodon complex: a monographic review: 553-571*. (Eds Gloyd, H. K. & Conant, R.). Society for the Study of Amphibians and Reptiles, Oxford, Ohio.
- Hayes, W. K., Kaiser, I. I. & Duvall, D. (1992). The mass of venom expended by prairie rattlesnakes when feeding on rodent prey. In *Biology of the pitvipers: 383-388*. (Eds Campbell, J. A. & Brodie, E. D.). Selva, Tyler, Texas.
- Klauber, L. M. (1972). *Rattlesnakes: their habits, life histories, and influence on mankind*. (2nd edn). University of California Press, Berkeley.

- Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Pääbo, S., Villablanca, F. X. & Wilson, A. C. (1989). Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc. natn. Acad. Sci. USA* 86: 6196–6200.
- Mackessy, S. P. (1988). Venom ontogeny in the Pacific rattlesnakes *Crotalus viridis helleri* and *C. v. oreganus*. *Copeia* 1988: 92–101.
- Manly, B. J. F. (1985). *The statistics of natural selection*. Chapman and Hall, London.
- Mayr, E. (1975). *Populations, species and evolution*. (Fourth printing). Harvard University Press, Cambridge, Massachusetts.
- McElroy, D., Morgan, P., Bermingham, E. & Kornfield, I. (1992). REAP: an integrated environment for the manipulation and phylogenetic analysis of restriction data. *J. Hered.* 83: 157–158.
- Mebs, D. (1978). Pharmacology of reptilian venoms. In *Biology of the Reptilia* 8: 437–560. (Eds Gans, C. & Gans, K. A.). Academic Press, London & New York.
- Moritz, C., Schneider, C. J. & Wake, D. B. (1992). Evolutionary relationships within the *Ensatina eschscholtzii* complex confirm the ring species interpretation. *Syst. Biol.* 41: 273–291.
- Plagnol, H. & Martin, P. (1957). Electrophorèse du venin d'*Ancistrodon rhodostoma* Boie. *Annls Inst. Pasteur, Paris* 92: 525–533.
- Pough, F. H. & Groves, J. D. (1983). Specializations of the body form and food habits of snakes. *Am. Zool.* 23: 443–454.
- Rael, E. D., Johnson, J. D., Molina, O. & McCrystal, H. K. (1992). Distribution of a Mojave toxin-like protein in rock rattlesnake (*Crotalus lepidus*) venom. In *Biology of the pitvipers: 163–168*. (Eds Campbell, J. A. & Brodie, E. D.). Selva, Tyler, Texas.
- Rice, W. R. (1989). Analyzing tables of statistical tests. *Evolution* 43: 223–225.
- Russell, F. E. (1983). *Snake venom poisoning*. Scholium International, New York.
- Salomão, M. G., Santos, S. M. A. & Puerto, G. (1995). Activity patterns of the rattlesnake *Crotalus durissus* (Viperidae, Crotalinae): feeding, reproduction and snakebite. *Stud. neotrop. Fauna Envir.* 30: 101–106.
- Sazima, I. (1992). Natural history of the jararaca pitviper, *Bothrops jararaca*, in southeastern Brazil. In *Biology of the pitvipers: 199–216*. (Eds Campbell, J. A. & Brodie, E. D.). Selva, Tyler, Texas.
- Shine, R. (1991). *Australian snakes: a natural history*. Reed Books, Balgowlah, New South Wales.
- Tan, N.-H. (1991). The biochemistry of venoms of some venomous snakes of Malaysia — a review. *Trop. Biomed.* 8: 91–103.
- Tan, N.-H., Ponnudurai, G. & Mirtschin, P. J. (1993). A comparative study of the biological properties of venoms from juvenile and adult inland taipan (*Oxyuranus microlepidotus*) snake venoms. *Toxicon* 31: 363–367.
- Tan, N.-H. & Tan, C. S. (1987). Thermal stability of venom enzymatic activities. In *Progress in venom and toxin research: 188–198*. (Eds Gopalkrishnakone, P. & Tan, C. K.). National University of Singapore, Singapore.
- Thomas, R. G. & Pough, F. H. (1979). The effect of rattlesnake venom on digestion of prey. *Toxicon* 17: 221–228.
- Thorpe, R. S. (1991). Clines and cause: microgeographic variation in the Tenerife gecko (*Tarentola delalandii*). *Syst. Zool.* 40: 172–187.
- Thorpe, R. S., Malhotra, A., Black, H., Daltry, J. C. & Wüster, W. (1995). Relating geographic pattern to phylogenetic process. *Phil. Trans. R. Soc. (B)* 349: 61–68.
- Tu, A. T. & Ganthavorn, S. (1978). Comparison of snake venoms (Reptilia, Serpentes) from Java, Indonesia and Thailand and its significance in evolution and zoogeography. *J. Herpet.* 12: 105–107.

**The role of ecology in determining venom variation in *Calloselasma rhodostoma* 171**

- Warrell, D. A. (1986). Tropical snake bite: clinical studies in South-East Asia. In *Natural toxins — animal, plant and microbial*: 25–45. (Ed. Harris, J. B.). Clarendon Press, Oxford.
- Williams, V., White, J., Schwaner, T. D. & Sparrow, A. (1988). Variation in venom proteins from isolated populations of tiger snakes (*Notechis ater niger*, *N. scutatus*) in South Australia. *Toxicon* 26: 1067–1075.
- Wüster, W. & Thorpe, R. S. (1991). Asiatic cobras: systematics and snakebite. *Experientia* 47: 205–209.